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(54) Title: OLIGONUCLEOTIDE-POLYAMIDE CONJUGATES

(57) Abstract

Oligonucleotide-polyamide conjugates of the formula X-L-Y, where X is a polyamide, Y is an oligonucleotide, and L is a linker which forms a covalent bond with the amino-terminus of the polyamide X and the 3' phosphate group of the oligonucleotide Y. Conjugates may be synthesized by assembling a polyamide on a solid support matrix, adding a suitable linker molecule, followed by synthesis of the oligonucleotide. Methods for detecting specific polynucleotides with oligonucleotide-polyamide conjugates are also described.

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OLIGONUCLEOTIDE-POLYAMIDE CONJUGATES

The present invention relates to oligonucleotide-
polyamide conjugates, their methods of production,
and the uses of such conjugates, particularly as
diagnostic and therapeutic reagents.

5   Synthetic oligonucleotides have found wide
application in the field of molecular biology,
particularly as hybridization probes for the
detection of DNA or RNA sequences. Generally, the
oligonucleotide carries a radiolabel at its 5' end to
10 enable detection of hybridization. Apart from the
problems normally associated with radiolabelled
materials such as cellular toxicity and mutagenicity,
the detection of radiolabelled probes requires
autoradiographic exposure, often for several days.
15 Additionally, radiolabels may have a short half-life,
thereby limiting their capacity for storage and
subsequent use.

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The labelling of oligonucleotides with
non-radioactive probes or reporter groups such as
fluorescent or enzymic reporter groups, offers
significant advantages over radiolabelled probes,
5 including greater safety, indefinite shelf life and
ease of detection.

It has been proposed to label oligonucleotides
with non-radioactive reporter groups through one or
more nucleotide bases (United States Patent No.
10 4,669,876 and published Australian Patent Application
No. 16484/85) or by directly coupling a reporter
group to the 3' or 5' ends of an oligonucleotide
(published European Patent Application Nos.
84101392.3 and 85102130.3). Such prior proposals
15 often involve complex synthetic reactions and may
disrupt the hybridization of the oligonucleotide to a
complementary target sequence.

A requirement accordingly exists for
oligonucleotides which hybridize efficiently to
20 complementary target sequences and which can be
conveniently detected without recourse to radioactive
labels, and further, which can be relatively simply
and conveniently prepared.

According to the present invention there is
25 provided an oligonucleotide-polyamide conjugate of
the formula X-L-Y, where X is a polyamide, Y is an
oligonucleotide, and L is a linker which forms a
covalent bond with the amino-terminus of the
polyamide X and the 3' phosphate group of the
30 oligonucleotide Y.

The polyamide may be formed from naturally
occuring amino acids (Biochemistry, 2nd Edition,
Albert L. Lehninger, pp. 72-77), such as lysine,
valine, glycine, serine, threonine, tyrosine,

methionine, proline, etc. linked through amide or
so-called peptide bonds. Alternatively, the
polyamide may be formed from synthetic amino acids
(that is, amino acids which do not occur naturally in
5   proteins) or a combination of natural and synthetic
amino acids. The term "synthetic amino acids" used
herein refers to α,ω-amino carboxylic acids which
may be represented by the general formula
$H_2NCHRCOOH$, where R is any organic moiety such as
10 alkyl or cycloalkyl which may unsaturated or partly
saturated and/or interrupted by one or more hetero
atoms or groups containing such hetero atoms such as
amide groups and/or substituted with halogen, cyano,
amino or unsubstituted or substituted phenyl or
15 benzyl.
        The polyamide may contain any number of amino
acid units, with the proviso of course that it does
not interfere with the hybridization of the
oligonucleotide with its target sequence. By way of
20 example only, the polyamide may contain from 1 to 100
amino acid units.
        The polyamide may form a peptide comprising
naturally occuring α-amino acids. The sequence of
the peptide can be designed to suit any desired
25 application of the oligonucleotide-polyamide
conjugate. The peptide may contain one or more
lysine residues which can be derivatized with a
reporter group as will be hereinafter described.
Furthermore, the polyamide may be antigenic, and thus
30 detectable by the binding of antibodies, which may,
for example, contain suitable reporter groups to
allow detection of antibody binding.
        Synthetic amino acids may, for example, be used
as spacers between amino acids such as lysine, which

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carry reporter groups, so as to avoid quenching or
steric inhibition of reporter groups, or to distance
a bulky reporter group from the oligonucleotide. An
example of a useful amino acid spacer is
5 6-aminohexanoic acid (Aha).

Synthetic amino acids which may be employed in
the invention include compounds of the formula:

$$\underset{RO}{\overset{O}{\diagdown}}C-(CH_2)_n W(CH_2)_{n_1} NHR^2$$

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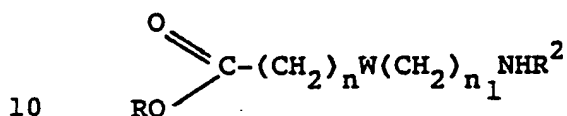
where: RO is a leaving group such as
         p-nitrophenyloxy, pentafluorophenyloxy and
         N'-hydroxysuccinimidyl; or R is hydrogen;
         R^2 is an amino protecting group such as
15   fluorenylmethoxycarbonyl (Fmoc) or tert-
         butoxycarbonyl (Boc);
         n and n_1 can be from 0 to 30;

$$W \text{ is } \left[-HN\overset{O}{\overset{\|}{C}}(CH_2)_{n2} \right] n_3 \text{ where } n_2 \text{ and}$$

20   n_3 may be from 0 to 100.

An amino acid unit useful in polyamide synthesis
is

$$\text{(pentafluorophenyl ring)} \underset{O}{\overset{O}{\diagdown}}C-(CH_2)_5 NH\overset{O}{\overset{\|}{C}}(CH_2)_5 NHFmoc$$

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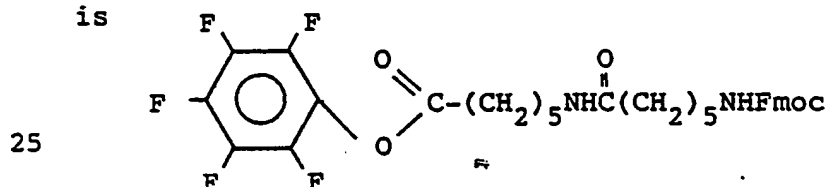
It is to be understood that the synthetic amino
acids which may be used to form the polyamide are in
no way restricted to the compound specifically
30 exemplified above.

The polyamide X may be labelled with one or more
reporter groups (also referred to as detectable
markers) such as biotin, fluorophores,
chemiluminescent moieties, enzymes or colloidal

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compounds such a ferritin or colloidal silver or gold.
     Fluorophore reporter groups may be selected from:
     fluorescein-5-isothiocyanate, diacyl (such as
isobutyryl, acetyl or pivaloyl) fluorescein-5 and/or
5   6 carboxylic acid pentafluorophenyl ester,
     6-(diacyl-5 and/or 6-carboxamido-fluorescein)amino-
     hexanoic acid pentafluorophenyl ester, Texas Red
     (Trademark of Molecular Probes, Inc.),
     tetramethylrhodamine-5 (and 6) isothiocyanate,
10   eosin-isothiocyanate, erythrosin- 5-isothiocyanate,
     4-chloro-7-nitrobenz-2-oxa-1,3-diazole,
     4-fluoro-7-nitrobenz-2-oxa-1,3-diazole,
     3-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) methylamino-
     propionitrile, 6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-
15   aminohexanoic acid, succinimidyl 12-(N-methyl-N-
     (7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminododecanoate,
     7-diethylamino-3-(4'-isothiocyanatophenyl)-4-methylcoum
     arin (CP), 7-hydroxycoumarin-4-acetic acid,
     7-dimethylaminocoumarin-4-acetic acid, succinimidyl
20   7-dimethylaminocoumarin-4-acetate, 7-methoxy-
     coumarin-4-acetic acid, 4-acetamido-4'-isothio-
     cyanatostilbene-2-2'-disulfonic acid (SITS),
     9-chloroacridine, succinimidyl 3-(9-carbazole)-
     propionate, succinimidyl 1-pyrenebutyrate,
25   succinimidyl 1-pyrenenonanoate, p-nitrophenyl
     1-pyrenebutyrate, 9-anthracenepropionic acid,
     succinimidyl anthracene-9-propionate,
     2-anthracenesulfonyl chloride; or fluorophore
     precursors, which when treated in a particular manner
30   fluoresce.
     Enzymic reporter groups may be selected from
     ß-galactosidase, horse radish peroxidase, urease,
     alkaline phosphatase, dehydrogenases, luciferase and
     carbonic anhydrase. Generally, enzymes will react

with one or more substrates to produce a detectable
signal such as a colour change, luminescence or
formation of a precipitate.

   Reporter groups may be attached to polyamides
5 according to conventional techniques known per se in
the art. For example, nucleophilic groups on
polyamides such as primary amine groups may react
with the fluorescent or enzymic reporter groups to
form a covalent bond therebetween. Alternatively,
10 bifunctional coupling reagents known per se in the
art (for example as described in the Pierce Cemical
Company catalogue , 1987) may be employed to attach
reporter groups to polyamides.

   Biotinylated oligonucleotides may be prepared by
15 conventional methods. For example, underivatised
biotin may be incorporated into an oligonucleotide
utilising BOP coupling methodology (Castro, B. et
al., Synthesis (1976), pp. 751-752). Alternatively,
biotin can be introduced as the N-hydroxysuccinimidyl
20 active ester. Biotin may be detected using avidin
attached to a reporter group. For example, a
streptavidin-alkaline phosphatase conjugate may be
employed to bind to biotin. The alkaline phosphatase
can react with a suitable substrate to generate an
25 insoluble dye precipitate which can be detected
visually.

   Where it is desired to detect the polyamide by
way of immunological reaction, antibodies directed
against the polyamide may be raised in suitable host
30 animals by immunization with oligonucleotide-
polyamide conjugates, the polyamide alone, or the
polyamide associated with a carrier molecule, for
example, KLH (key hole limpet haemocynanin),
according to methods well known in the art (for

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example, Goding, J.W. (1986), Monoclonal Antibodies:
Principles and Practice, 2nd Edition, Academic Press).

   The oligonucleotide may be of any desired
sequence which allows hybridization to a
5 complementary nucleotide sequence in a DNA or RNA
target. The number of nucleotides which constitutes
the oligonucleotide is generally unimportant, as long
as sufficient nucleotides are present to allow
hybridization to a target sequence. Usually, the
10 oligonucleotide will contain in excess of six
nucleotides. The oligonucleotide may be suitably
modified to increase its half-life in-vivo without
effecting hybridization. For example, the
oligonucleotide may be modified by replacing one or
15 two of the non-bridging oxygens on the phosphorous
backbone with sulphur or amines, according to the
procedures of Argawal et al. (1988, Proc. Natl. Acad.
Sci. USA 85, pp. 7079-7083) or Stein and Cohen,
(1988, Cancer. Res. 48, pp. 2659-2688). Such
20 modified oligonucleotides are within the scope of the
term oligonucleotide. The term "oligonucleotide" may
also include a single nucleotide.

   The linker L refers to a moiety derived from a a
bifunctional molecule R'-L'-R", wherein R' and R"
25 are the same or different and represent such
functional groups as -NH$_2$, -CO$_2$H, -OH, OR where R
is a hydroxy protecting group, -CO$_2$R, where R is
2-hydroxypyridine, N-hydroxysuccinimide, p-nitro-
phenyl, pentafluorophenyl (Pfp), Me or other active
30 esters, acylimidazole, maleimide, trifluoroacetate,
diketone, imidoesters, sulfonate esters, imine, -CHO,
1,2-cyclohexanedione, glyoxal, sulfonyl halides,
alpha halo ketones, azide, etc, and L is an alkyl or
substituted alkyl group. Alkyl chain L can be

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substituted with such common substituents as halogen,
(I, Br, Cl, F), hydroxy, cyano, phenyl, amino,
carboxy, alkyl, alkoxy and others. Further, the
alkylene chain of linker L can be interrupted by one
5   or more bivalent groups, such as -O-, -S-, -NH-
-CH=CH-, -C=C-, phenyl, $-SO_2-$, etc. However,
functional group R' must be capable of forming under
appropriate conditions, a covalent bond with the
amino terminus of a polyamide and functional group R"
10 must be capable of forming, under appropriate
conditions, a covalent bond with a nucleotide.
Clearly the choice of linking group R'-L-R" and a
particular conjugation chemistry must reflect the
need to preserve other macromolecular bonds critical
15 to the integrity of the resulting probe molecule,
i.e. peptide, glycosidic and phosphodiester bonds.
       The linker may be derived from an α,ω hydroxy
carboxylic acid derivative.
       Alternatively the linker L may be a bond, or a
20 lactone such as butyrolactone.
       .The present invention also provides a method for
the production of an oligonucleotide-polyamide
conjugate comprising the step of linking the
3'-terminal end of an oligonucleotide to the amino
25 terminus of a polyamide.
       Thus, in accordance with one aspect of the
method, the conjugate may be produced by attaching a
linking group to either a preformed oligonucleotide
moiety or a preformed polyamide moiety and then
30 attaching the remaining moiety to the linking group.
       Alternatively, appropriate linking group
precursors may be attached to the preformed
oligonucleotide moiety and the preformed polyamide
moiety. Reaction of the two precursors then results

in the formation of the linking group.

In accordance with a further aspect, the method comprises attaching the linking group to a preformed polyamide and thereafter attaching a nucleotide base to the linking group and then sequentially adding one or more further nucleotide bases to form an oligonucleotide.

In a still further aspect, the method comprises attaching the linking group to a preformed oligonucleotide and thereafter attaching an amino acid to the linking group and then sequentially adding one or more amino acids to form a polyamide.

The polyamide moiety may be connected to a solid support matrix such as controlled pore glass (CPG).

In accordance with a particularly preferred aspect of the present invention, there is provided a method for the production of an oligonucleotide-polyamide conjugate, said method comprising the steps of:

(a)   reacting the C-terminus of a first amino acid or a unit of amino acids (linked together by amide bonds) with a support matrix to form a bond therebetween;

(b)   thereafter sequentially reacting the support matrix with one or more amino acids, according to well-known solid-phase peptide synthetic techniques to form a polyamide;

(c)   reacting the support matrix-polyamide with a first reactive group of a linker to form a bond between the amino terminus of the polyamide and the linker;

(d)   reacting the product of step (c) with a first nucleotide to form a bond between a second reactive group on the linker and the 3' phosphate of

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the nucleotide;

(e)   thereafter sequentially reacting the support
matrix with one or more nucleotides, according to
well-known solid phase oligonucleotide synthetic
5   methods to form an oligonucleotide; and

(f)   optionally cleaving the
oligonucleotide-polyamide conjugate from the support
matrix and removing any protecting groups associated
with reactive groups of the polyamide or
10   oligonucleotide, and purifying the resulting
conjugate.

When the linker L is a bond, step (c) is omitted.

Polyamides may, for example, be synthesized using
solid phase Fmoc (Atherton, R. and Shepard, R.C.
15   (1985) .J. Chem. Soc. Commun, pp. 165-166) or solid
phase Boc (Barany, G. and Merrifield, R.B. (1980)
Solid-Phase Peptide Synthesis in "The Peptides", Vol.
2, E. Gross & J. Meienhofer Eds., Academic Press, New
York, pp. 1-284) methodologies. In these methods,
20   the amino acids are protected with standard
protecting groups known per se in the art (for
example, Green (1981) Protective Groups in Organic
Synthesis, John Wiley & Sons, Inc.; Atherton and
Sheppard (1985) J. Chem. Soc. Commun, pp. 165-166;
25   Barany and Merrifield, Supra) to protect reactive
moieties.

Oligonucleotides may be synthesized by the solid
phase phosphotriester method (Sproat and Gait (1984)
Oligonucleotide Synthesis, A Practical Approach, pp.
30   83-116, IRL Press, Oxford), solid phase H-phosphonate
method (Froehler et al. (1986) Nucleic Acids
Research, 14, pp. 5399-5407) or the solid phase
phosphoramidite method (Beaucage and Caruthers (1981)
Tetrahedron Lett., 22, pp. 1859-1862). In each of

these methodologies, reactive groups such as hydroxy
or amino groups may be protected with standard
hydroxy and amino protecting groups as described by
(Green (1981) Protective Groups in Organic Synthesis,
5 John Wiley & Sons, Inc.; Beaucage, S.L. and
Caruthers, M.H. (1981) Tetrahedron Lett., 22, pp.
1859-1862; Sproat, S. and Gait, M.J. (1984)
Oligonucleotide Synthesis, A Practical Approach, pp.
83-116, IRL Press, Oxford).
10   On completion of synthesis of the
oligonucleotide-polyamide conjugate, deprotection may
be carried out according to methods known per se in
the art.
       The oligonucleotide-polyamide conjugate of the
15 present invention may be fully protected and attached
to a support matrix, protected form detached from the
support matrix, or in a fully deprotected form. Each
of these states are within the scope of the term
"oligonucleotide-polyamide conjugate".
20   The support matrix may, for example, be selected
from controlled pore glass such as aminopropyl
controlled pore glass (AP-CPG) or polystyrene
resins. The support matrix coupled to the polyamide
constitutes a solid support upon which
25 oligonucleotide synthesis takes place.
       The preferred method of synthesis of the
oligonucleotide-polyamide conjugates of the present
invention is advantageous in that large batches of
the polyamide connected through its C-terminal to the
30 support matrix may be prepared in advance and
aliquots used for the assembly of a desired
oligonucleotide when required. Additionally,
stepwise synthesis results in excellent yields of the
oligonucleotide-polyamide conjugate, which are

gr ater than those achieved by coupling a preformed
oligonucleotide to a preformed polyamide.

The polyamide may be synthesized in a standard
commercial peptide synthesizer (available, for
5 example, from Applied Biosystems Inc.), and then
transferred to a standard commercial oligonucleotide
synthesizer (such as supplied by Applied Biosystems
Inc.) for synthesis of the oligonucleotide.

One or more reporter groups may be introduced
10 into the polyamide at a number of different stages.
The reporter group can be present in the amino acids
prior to polyamide synthesis (stage I); it can be
introduced after polyamide synthesis (stage Ia);
after addition of the linker (stage II); after
15 oligonucleotide synthesis on the support matrix
(stage III); or after deprotection and purification
of the oligonucleotide-polyamide conjugate from the
support matrix (stage IV). The method chosen will
depend upon the choice of reporter groups and
20 synthetic procedure.

.If the reporter group is stable to the conditions
of both peptide and oligonucleotide synthesis, it can
be incorporated from the start of polyamide
synthesis, as a derivatized amino acid (stage Ia).
25 If it is stable to the conditions of DNA synthesis
but not those of peptide synthesis, it can be
incorporated after the polyamide has been synthesized
(stages I or II). If the reporter group is not
stable to either peptide or oligonucleotide chain
30 assembly, but is stable to deprotection methods, it
can be incorporated after oligonucleotide chain
assembly of the fully protected polyamide-
oligonucleotide conjugate (stage III). These methods
are convenient, since the label is being introduced

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while the polyamide-oligonucleotide conjugate is
still on the solid support, and thus an excess of
reporter group can be used and the excess simply
washed away after reaction. If the label is not
5   stable to any of the conditions used in the synthesis
of the conjugates, it can be introduced in a solution
phase reaction with the purified fully deprotected
polyamide-oligonucleotide conjugate (stage IV).
      Fluorophores may be introduced into the
10 oligonucleotide-polyamide conjugate at any of stages
I to IV. This is also the case for biotin.
      Enzymes, and colloidal compounds such as
colloidal gold, colloidal silver, or ferritin may be
introduced at stage IV.
15   The polyamide portion of the oligonucleotide-
polyamide conjugate may contain multiple reporter
groups which may increase the detectable signal
produced therefore facilitating detection.
      The polyamide portion of the conjugate not only
20 functions as a vehicle for attaching a reporter
group, but may also act as an address marker to
target a polyamide to a particular cell type,
cellular location, or enhance the passage of an
oligonucleotide through a cellular membrane. The
25 address label activity of peptide sequences is well
established (Verner and Schatz (1988) Science 241,
pp. 1307-1313; and Goldfarb et al. (1986) Nature 322,
pp. 641-644). By selecting a peptide sequence which
is, for example, recognised by a cell surface
30 receptor, oligonucleotides conjugated to that peptide
sequence may be transported into specific cell types
where they can exert a biological effect, such as, in
the case of anti-sense oligonucleotides, blocking
transcription of viral or cellular RNA.

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In another aspect of the invention there is
provided a method for the treatment of viral,
bacterial or other diseases, characterised in that a
patient in need of such treatment is administered a
5 therapeutically effective amount of an
oligonucleotide-polyamide conjugate of the present
invention, and further characterised in that the
oligonucleotide portion of the conjugate is an
anti-sense oligonucleotide which is complementary to
10 a specific viral, bacterial or other polynucleotide,
such that transcription or translation of the
specific polynucleotide is blocked.
    The hybridization of anti-sense oligonucleotides
to specific target polynucleotides may inhibit the
15 synthesis of viral or bacterial proteins associated
with viral or bacterial integrity or propagation,
and/or the synthesis of toxins.
    The reference to "other diseases" pertains to
disease states due to expression of genes endogenous
20 to a cell. For example, mRNA encoding a protein
causing or contributing to cellular transformation
such as the myc protein, may be inactivated with the
polyamide-oligonucleotide conjugates of the invention
bearing an appropriate anti-sense oligonucleotide.
25   A therapeutically effective amount of an
oligonucleotide is that which blocks transcription or
translation of a desired polynucleotide. This amount
will vary according to the abundance of the desired
polynucleotide and/or its rate of synthesis, the rate
30 of cellular uptake and/or stability of the conjugate,
and the weight and age of the subject being treated.
In each case, what constitutes a therapeutically
effective amount would be based on a determination
made by a consulting physician or veterinarian.

The polyamide moiety of the oligonucleotide-
polyamide conjugates may also act to stabilise the
oligonucleotide moiety from cellular degradation (Le
Maitre et al. (1987) Biochemistry 84; pp. 648-652).

5   Alternatively, the oligonucleotide moiety may
enhance the properties of the polyamide, such as, for
example, improving its solubility.

According to a yet further aspect of the present
invention, there is provided a polyamide having the
10   formula Z-X-L, where Z represents a solid phase
matrix, X represents a polyamide linked through its
C-terminus to the solid phase matrix, and L
represents a bifunctional linker having a first
reactive group which is attached to the N-terminus of
15   the polyamide, and a second reactive group which is
capable of forming a bond with the 3' phosphate of a
nucleotide.

This polyamide is an intermediate in the
synthesis of the conjugates of the present
20   invention. An oligonucleotide may be directly
synthesized on the second reactive group of the
bifunctional linker L.

The conjugates of the present invention have
considerable utility as hybridization probes for the
25   detection of specific DNA or RNA sequences in a
target sample. Binding of the oligonucleotides to
target sequences is detected by reporter groups
attached to the polyamide or by antibodies which bind
to the polyamide. The conjugates of the present
30   invention may therefore be used in the detection of
viral nucleic acids, such as the AIDS virus or
Hepatitis virus, bacterial nucleic acids, or DNA
associated with genetic disorders such as muscular
dystrophy or cystic fibrosis. The conjugates of the

invention may be employed in hybridization to target
sequences bound to a matrix such as nitrocellulose,
derivatized paper or nylon membranes. Alternatively,
the conjugates may be employed in in-situ
5   hybridization (also known as "hybridization
histochemistry") to tissue sections to detect target
polynucleotides within the tissue section.

In another aspect of the invention there is
provided a method for detecting the presence or
10   absence of a specific viral, bacterial or other
polynucleotide in a biological sample comprising
contacting the nucleic acids of the sample with an
oligonucleotide-polyamide conjugate of the present
invention wherein the oligonucleotide portion of the
15   conjugate is complementary to a portion of a target
polynucleotide, and thereafter detecting whether
hybridization of the conjugate has occurred.

A biological sample may comprise a biological
fluid such as blood or plasma; cells, such as
20   lymphocytes; or a tissue biopsy. Nucleic acids, that
is, .DNA or RNA, from the sample may be extracted and
detected with the conjugate, according to methods
known per se in the art. The conjugate may itself be
labelled with reporter groups for detection of
25   hybridization, or alternatively, conjugate bound to a
target polynucleotide may be suitably detected, with,
for example, antibody reagents.

In a further aspect of the invention there is
provided a method for detecting a specific
30   polynucleotide immobilized to or otherwise associated
with a support matrix, said method comprising
contacting the support matrix with an
oligonucleotide-polyamide conjugate according to the
present invention, wherein the oligonucleotide

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portion of the conjugate is complementary to a
portion of the target polynucleotide, and thereafter
detecting hybridization of the conjugate to the
support matrix.

5   In another aspect of the invention there is
provided a method for determining the presence and
location in animal or plant tissue of a specific
polynucleotide population which comprises:

(a)   preparing a section of the tissue to be
10   examined;

(b)   hybridizing the tissue section with an
oligonucleotide-polyamide conjugate according to the
present invention, wherein the oligonucleotide
portion of the conjugate is complementary to a
15   portion of a target polynucleotide;

(c)   removing unhybridized probe material from
the tissue section; and

(d)   detecting or identifying the locations in
the tissue section where labelling by hybridization
20   of the conjugate has occurred.

. In yet a further aspect of the invention there is
provided a diagnostic kit for detecting a desired
polynucleotide, which comprises an oligonucleotide-
polyamide conjugate according to the present
25   invention, wherein the oligonucleotide portion of the
conjugate is complementary to a portion of the
desired polynucleotide; and reagents for detecting
hybridization of the conjugate.

Such a diagnostic kit may additionally comprise
30   reagents for tissue section preparation. Examples of
such reagents are formaldehyde, glutaraldehyde and
acetic acid.

The present invention will now be further
described with reference to the following

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non-limiting Examples and Figures, in which:

FIGURE 1 shows a reaction scheme for solid phase peptide synthesis;

FIGURE 2 shows a reaction scheme for the
5   preparation of an oligonucleotide-polyamide conjugate;

FIGURE 3 shows an Electrophoretic analysis of the crude KPIB-(AlaLys)$_5$-Ala (lane 1) and normal KPIB (lane 2) end labelled with ^{32}P, on a denaturing 20% polyacrylamide gel;

10   FIGURE 4 shows a dot blot hybridization using biotin labelled polyamide-oligonucleotide conjugates. NC refers to negative control; and

FIGURE 5 shows a biotin labelled polyamide-kallikrein oligonucleotide hybridizing to a
15   6µm frozen section of male mouse submandibular gland. Dark areas show hybridization of the conjugate.

EXAMPLE 1:
20   Synthesis of Reagents:

.4-Nitrophenyl 4-(4,4'-dimethoxytrityloxy)-butyrate (**1a**). Sodium 4-hydroxybutyrate (1.26 g, 10 mmol) and 4,4'-dimethoxytrityl chloride (DMTrCl) (3.39 g, 10 mmol) were stirred in 30 mL of pyridine
25   for 16 h. 4-nitrophenol (1.30 g, 10 mmol) and dicyclohexylcarbodiimide (DCC) (2.06 g, 10 mmol) were added and stirred for a further 2 days. The reaction mixture was filtered, and the solution was then concentrated and flash chromatographed on 70 g of
30   silica gel with 25% EtOAc/petroleum either to give a light yellow oil (5.0 g, 95%). ^{1}H NMR (CDCl$_3$) δ 2.04 (m, 2H, H$_3$), 2.7 (t, J=7.2 Hz, 2H, H$_2$), 3.2 (t, J=5.9 Hz, 2H, H$_4$)., 3.77 (s, 6H, OCH$_3$), 6.8-7.5 (m, 15H, ArH) 8.2 (d, J=9.2 Hz, 2H, PhNO$_2$

m-H). ^{13}C NMR (CDCl$_3$) δ 25.2 (C3), 31.6 (C2),
55.2 (OCH$_3$), 62.0 (C4), 86.0 (CAr$_3$), 113.1 126.8,
127.1, 127.8, 127.9,128.1, 129.1, 130.0, 136.2,
145.0, 158.4 (DMTr), 122.4, 125.1, 145.2, 155.4,
5 (PhNO$_2$), 171.1 (CO$_2$). The product contained some
EtOAc solvent impurity.

4-Nitrophenyl 4-[(9-phenyl-9-xanthyl)oxy]-
butyrate (1b). This compound was synthesized using
the same method used to synthesize 1a except that
10 pixyl chloride (PxCl) was substituted for
dimethoxytrityl chloride, to give 1b in 80% yield, mp
130-130.5°C (EtOAc). ^{1}H NMR (CDCl$_3$) δ 1.98 (m,
2H, H3), 2.7 (t, J=7.3 Hz, 2H, H2), 3.0 (t, J=5.8 Hz,
2H, H4), 7.0-7.5 (m, 15H, ArH), 8.2 (d, J=7.1 Hz, 2H
15 PhNO$_2$ m-H). ^{13}C NMR (CDCl$_3$) δ 25.0 (C3),
31.5 (C2), 61.8 (C4), 75.4 (CPh$_3$), 116.3, 123.2,
123.5, 126.4, 126.6, 127.9, 129.1, 129.4, 148.9,
151.3 (Px C), 122.4, 125.1, 145.2, 155.4 (PhNO$_2$ C),
171.0 (CO$_2$). Anal. Calcd for C$_{29}$H$_{23}$NO$_6$: C,
20 72.3; H, 4.8; N, 2.9. Found: C, 72.0; H, 4.4; N,
3.2.

4-Nitrophenyl 3-[6-(4,4'-dimethoxytrityloxy)-
hexylcarbamoyl]propanoate(2). A solution of
succinic anhydride (1.0 g, 10 mmol) and
25 6-aminohexanol (1.17 g, 10 mmol) in pyridine (10 mL)
was stirred for 4 d. DMTrCl (3.39 g, 10 mmol) was
then added, it was stirred for a further 4 h, and
p-nitrophenol (1.39 g, 10 mmol) and DCC (2.06 g, 10
mmol) were then added and the mixture was stirred for
30 a further 2 d. The reaction mixture was filtered,
the solution concentrated and flash chromatographed
on 100 g of silica gel with 50% EtOAc/petroleum eith-
er to give a light yellow oil (4.09 g, 64%). ^{1}H
NMR (CDCl$_3$) δ 1.2-1.7 (m, 8H, CH$_2$), 2.5

20

(t, J=6.5, Hz, 2H, CH_2), 2.93 (t, J=6.5 Hz, 2H,
CH_2), 3.01 (t, J=6.4 Hz, 2H, CH_2), 3.2 (t, J=6.4
Hz, 2H, CH_2), 3.76 (s, 6H, OCH_3), 6.8-7.5 (m,
15H, ArH), 8.2 (d, J=9.2 Hz, 2H, $PHNO_2$ m-H). [13]C
NMR ($CDCl_3$) δ 25.83, 26.69, 29.53, 29.87, 30.55
(CH_2), 39.68 (CH_2NHCO), 55.14 (OCH_3), 63.16
($DMTrOCH_2$), 85.60 (CAr_3), 112.91, 126.53, 127.64,
127.70 127.79, 128.10, 129.07, 129.95, 136.60,
145.32, 158.26, (DMTr C), 122.4, 125.1, 145.31, 155.8
($PhNO_2$ C), 170.49, 170.75 (C=O). The compound
contained some EtOAc solvent impurity that could not
be easily removed.

Pentafluorophenyl N-fluorenylmethoxycarbonyl-
6-aminohexanoate (FmocAhaOPfp, 3). 6-Aminohexanoic
acid (2.62 g, 20 mmol) and Na_2CO_3 (5.30 g, 50
mmol) were dissolved in 60 mL of H_2O, 25 mL of
dioxan was then added, followed by N-(Fluorenyl-
methoxycarbonyloxy)succinimide (Fmoc-NHS) (6.75 g, 20
mmol). The mixture was stirred vigorously for 16 h.
The cloudy reaction mixture was then poured into 1.2
L of H_2O to give a clear solution. This was
extracted with EtOAc (2 x 300 mL) and the aqueous
layer acidified to pH 3 using approximately 10 mL of
conc. HCl, to give a voluminous precipitate. This
was kept at 4°C for 16 h, and was then filtered to
give 6.05 g (86%) of FmocAhaOH.

To a solution of FmocAhaOH (1.77 g, 5 mmol) and
pentafluorophenol (Pfp 1.01 g, 5.5 mmol) in 8 mL of
DMF was added a solution of DCC (1.03 g, 5 mmol) in 2
mL of DMF. This was stirred for 16 h, filtered, and
the filtrate evaporated in vacuo to dryness giving
the crude ester which was recrystallized from 95%
EtOH/1% AcOH (approximately 10 mL) to give 2.41 g
(93%) of white needles, mp 128-129°C. ^{1}H NMR

(CDCl$_3$) δ 1.4 - 1.8 (m, 6H, CH$_2$), 2.7 (t, J=7.2
Hz, 2H, CH$_2$CO$_2$), 3.2 (m, 2H, NHC_H$_2$), 4.2 (5,
J=6.7 Hz, 1H, Fmoc CH), 4.4 (d, J=6.8 Hz, 2H, Fmoc
CH$_2$), 7.3-7.8 (m, 8H, Fmoc ArH). ^{13}C NMR

5   (CDCl$_3$) δ 24.36 (C4), 25.93 (C3), 29.62 (C5),
33.18 (C2), 40.73 (C6), 47.31 (Fmoc CH), 66.54 (Fmoc
CH$_2$), 119.98, 125.00, 127.02, 127.67 (Fmoc Aromatic
CH), 141.34, 143.99 (Fmoc Aromatic C), 156.46 (Fmoc
C=O), 169.36 (ester CO$_2$). Anal. Calcd for

10   C$_{27}$H$_{11}$NO$_4$F$_5$: C, 63.8; H, 2.2; N, 2.8. Found:
C, 63.9; H, 1.8; N, 3.2.

      N-(N-fluorenylmethoxycarbonyl-6-aminohexanoyl)-6-
aminohexanoic acid (FmocAha$_2$OH, 6). To a solution
of FmocAhaOH (prepared as above, 1.77 g, 5 mmol) and

15   N-hydroxysuccinimide (0.575 g, 5 mmol) in 8 mL of DMF
was added a solution of DCC (1.03 g, 5 mmol) in 2 Ml
of DMF. It was allowed to stir for 16 h, filtered,
and the filtrate evaporated in vacuo to a syrup.
This was recrystallized from isopropanol (~10 mL) to

20   give 1.94 g (86%) of 5.
      · To a solution of 5 (0.912 g, 2 mmol) in 10 mL
dioxan was added dropwise a solution of
6-aminohexanoic acid (0.524 g, 4 mmol) and Na$_2$CO$_3$
(0.424 g, 4 mmol) in 10 mL of. H$_2$O. The resulting

25   suspension was stired vigorously for 48 h, and was
then poured into 100 mL of H$_2$O to give a clear
solution. The pH of this rapidly stirring solution
was reduced to 3 by the dropwise addition of 10 ml
at 1 M KHSO$_4$. A voluminous precipitate formed, and

30   was kept at 4°C for 24 h, and then filtered to give a
quantitative yield of the acid. This crude product
was recrystallized from EtOAc to give 0.679 g (73%)
of a white powder, mp 106.5-107°C. ^{1}H NMR
(CD$_3$OD) δ 1.3-1.7 (m, 12H, internal CH$_2$), 2.1

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(t, J = 7.4 Hz, 2H, CH_2CONH), 2.3 (t, J = 7.3 Hz,
2H, CH_2CO_2H), 3.0-3.2 (m, 4H, $FmocNHCH_2$ and
$CH_2CONHCH_2$), 4.2 (t, J=6.8 Hz, 1H, Fmoc CH), 4.3
(d, J=6.8 Hz, 2H, Fmoc CH_2), 7.2-7.8 (m, 8H, Fmoc

5 ArH). Anal. Calcd for $C_{27}H_{34}N_2O_5$: C, 69.5;
H, 7.4; N, 6.0. Found: C, 69.2; H, 7.3; N, 5.8.

Pentafluorophenyl N-(N-Fluorenylmethoxycarbonyl-
6-aminohexahoyl)-aminohexanoate (FmocAha$_2$OPfp,
4). To a solution of 6 (233 mg, 0.5 mmole) and

10 pentafluorophenol (101 mg, 0.55 mmole) in 1 mL of DMF
was added DCC (103 mg, 0.5 mmole). This was allowed
to stir for 2d, and then filtered. The filtrate was
evaporated in vacuo to a creamy solid which was
recrystallized from 95% ETOH/1% AcOH (1 mL) to give

15 180 mg (57%) of pure 4, mp 126-127°C.

Pentafluorophenyl N-tert-butoxycarbonyl-6-
aminohexanoate (8). To a solution of
N-Boc-6-aminohexanoic acid (4.78 g, 20.8 mmol) and
pentafluorophenol (3.68 g, 20 mmol) in 50 mL of EtOAc

20 was added DCC (4.12 g, 20 mmol). This was allowed to
stir for 16 h, and was then filtered and the filtrate
evaporated in vacuo to a syrup. On standing, this
crystallized to give 7.46 g (94%) of the ester.
Rerystallization from isopropanol/1% acetic acid gave

25 6.26 g of white needles, mp 81-83°C. ^{1}H NMR
(CDCl$_3$) δ 1.4-1.6 (m, 15H, Boc CH_3 and internal
CH_2), 1.8 (m, 2H, $\underline{CH_2}CH_2CO_2$), 2.67 (5, J=7.3
Hz, 2H, CH_2CO_2), 3.1 (m, 2H, $NH\underline{CH}_2$), 4.5 (b,
1H, NH). ^{13}C NMR (CDCl$_3$) δ 24.38 (C4), 26.00

30 (C3), 28.39 (Boc CH_3), 29.70 (C5), 33.20 (C2),
40.28 (C6), 79 (Boc C), 156 (Boc C=O), 169 (C1).
Anal. Calcd for $C_{17}H_{20}NO_4F_5$: C, 51.4; H, 5.1;
N, 3.5. Found: C, 51.5; H, 5.0; N, 3.4.

Pentafluorophenyl N-(N-tert-butoxycarbonyl-6-

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Pentafluorophenyl N-(N-_tert_-butoxycarbonyl-6-
aminohexanoyl)-6-aminohexanoate (BocAha$_2$OPfp, _2_).
To a solution of 6-aminohexanoic acid (1.31 g, 10
mmol) in 5 mL of 1 M NaOH (5 mmol) and 5 mL H$_2$O was

5 added a solution of _8_ (1.99 g, 5 mmol) in 10 mL of
dioxan. The resulting fine suspension was stirred
vigorously for 3 d, by which time it was clear. This
solution was added to 200mL of H$_2$O, and the pH
decreased to 3.5 by the dropwise addition of

10 approximately 10 mL of 1 M KHSO$_4$, the resulting
solution extracted with EtOAc (3 x 100 mL), dried
(Na$_2$CO$_4$), and concentrated in vacuo to 3 mL.
Another 10 mL of EtOAc was then added, followed by
DCC (1.03 g, 5 mmol). It was stirred for 16 h, was

15 filtered, the filtrate evaporated to dryness in
vacuo, and the product was recrystallized from
EtOH/H$_2$O containing 1% AcOH (approximately 10 mL),
to give 1.75 g (67%) of white needles, mp
88-89°C. ^{1}H NMR (CDCl$_3$) δ 1.3-1.9 (m, 2H,

20 CH$_2$ and Boc CH$_3$), 2.2 (t, J=7.5 Hz, 2H,
C̲H̲$_2$CONH), 2.7 (t, J=7.3 Hz, 2H, CH$_2$CO$_2$), 3.1
(m, 2H, Boc NHC̲H̲$_2$), 3.3 (m, 2H, CONHC̲H̲$_2$), 4.6
(bs, 1H, Boc NH), 5.6 (bs, 1H, CONH). ^{13}C NMR
(CDCl$_3$) δ 24.35, 25.31, 26.10, 26.41, 29.31,

25 29.83, 33.17, 36.62, 39.14, 40.35 (CH$_2$), 28.44 (Boc
CH$_3$), 79.12 (Boc central C), 156.05 (Boc C=0),
169.39 (ester C=0), 172.84 (amide C=0). Anal. Calcd
for C$_{23}$H$_{31}$N$_2$O$_5$F$_5$:C, 54.1; H, 6.1; N, 5.5.
Found: C, 53.9; H, 5.9; N, 5.8.

30 Diisobutyryl-5(and 6)-carboxyfluorescein, penta-
fluorophenyl ester (_7_). Trimellitic anhydride (9.6
g, 0.05 mol) and Resorcinol (11 g, 0.11 mol) were
mixed thoroughly and placed in a 190°C oil bath for 1
h. The temperature was then increased to 210°C, and

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was kept there for 5 h, by which time the melt had
solidified to a dark red solid. It was then allowed
to cool down and dissolved in 50 mL of DMF. To this
solution was added 100 mL of pyridine and isobutyryl

5   chloride (15.4 mL, 0.15 mol), which was allowed to
stir for 24 h. Following filtration, the resulting
thick syrup was redissolved in 40 mL of EtOAc, washed
with 1 M H_2SO_4 (2 x 300 mL) and H_2O (1 x 300
mL), dried (Na_2SO_4), and again concentrated to a

10   syrup. It was redissolved in CH_2Cl_2 and flash
chromatographed on a silica gel column (170 g),
eluting first with CH_2Cl_2 (800 mL) and then with
2% MeOH/CH_2Cl_2. The fractions containing the
product were pooled and the solvent removed in vacuo

15   to give 12.45 g (48%) of crude diisobutyryl-5(and
6)-carboxyfluorescein.

        5.77 g of this material (11 mmol) and penta-
fluorophenol (2.32 g, 21.1 mmol) were dissolved in
125 mL of 1/1 EtOAc/CH_2Cl_2. The solution was

20   cooled down to 4°C, and DCC (2.5 g, 12.1 mmol) was
added dropwise. It was allowed to stir for 2 h, then
filtered and the solvent removed in vacuo to give an
oil, which solidified overnight at -20°C. This was
redissolved in 50 mL of CH_2Cl_2 and flash

25   chromatographed on a 150 g of silica gel, eluting
with CH_2Cl_2. The fractions containing the
product were pooled, the solvent removed in vacuo and
the product recrystallized from 95% EtOH/2% Acetic
acid, to give 3.68 g (59%) of white needles, mp

30   184-188°C. On tlc (CH_2Cl_2) this gave two closely
running spots, corresponding to the two isomers, at
Rf 0.58 and 0.63. ^{1}H NMR ($CDCl_3$) δ 1.31 (d,
J=6.9 Hz, 12H, CH_3), 2.8 (septet, J=7.0 Hz, 2H,
$\underline{CH}(CH_3)_2$), 6.83 (s, 4H, H_1' and H_2'), 7.11

(s, 2H, H_4'), 7.38 (d, J=8.0 Hz, 0.5H, H_7 of

5-isomer), 7.96 (s, 0.5H, H_7 of 6-isomer), 8.20 (d,

J=8.0 Hz, 0.5H, H_6 of 5-isomer), 8.5 (m, 1H, H_4

and H_5 of 6-isomer), 8.87 (s, 0.5H, H_4 of

5 5-isomer. ^{13}C NMR (CDCl$_3$) 18.82, 34.18, 82.11,

82.24, 110.61, 115.07, 115.11, 118.00, 125.01,

125.89, 126.36, 127.05, 128.08, 128.71, 129.28,

130.92, 132.23, 133.30, 136.32, 137.05, 138.2,

139.62, 141.8, 142.84, 151.46, 151.51, 152.66,

10 153.41, 158.21, 161.06, 167.50, 174.94. Anal. Calcd

for $C_{35}H_{23}F_5O_9$: C, 61.6; H, 3.4. Found: C,

61.5; H, 3.0.

   Since this material was analytically pure and was

the material used in the fluorescein labelling

15 experiments, it was used to determine the spectral

characteristics of carboxyfluorescein (CF). A 1 μM

solution of $\underline{7}$ in 0.1 M NH$_4$OAc, pH 9.0 was allowed

to stand for 3 d to decompose $\underline{7}$ to the CF nucleus,

and spectral measurements taken. The extinction

20 coefficient of this solution at 495 and 496 nm was

75000 M^{-1}, and at 260 nm 23000 M^{-1}. The

extinction coefficient of the released

pentafluorophenoxide ion in the same solution at 260

nm is very low (170 M^{-1}), which is within the error

25 limits of the absorption coefficient above, so it is

not taken into account. This solution was also used

as the standard reference for the fluorescence yields

of fluorescein containing polyamide-oigonucleotide

hybrids.

30 ^1H and ^{13}C NMR spectra were recorded on a

Bruker AM300 at 300 and 75 MHz respectively with

tetramethylsilane as internal reference.

Dimethylformamide (DMF) was distilled under reduced

pressure and used within 1-2 days. Pyridine was

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distilled from potassium hydroxide and stored over 5A
molecular sieves. Melting points were determined in
open ended capillaries on an Electrothermal Melting
Point Apparatus. Flash chromatography was carried
5 out using Merck Kieselgel # 9385.

   EXAMPLE 2:
        Peptide Synthesis:
        Fmoc (Fluorenylmethoxycarbonyl) peptide synthesis
10 methodology was used to synthesize a peptide
   containing multiple lysine residues that could
   subsequently be used as attachment sites for
   non-radioactive labels, and alanine residues that ·
   served as spacers between the lysine residues. This
15 peptide has the sequence (AlaLys)$_5$Ala. Controlled
   Pore Glass (CPG) was used as the solid support matrix
   for peptide synthesis, even though this support is
   not common in peptide synthesis, because it is the
   support of choice for oligonucleotide synthesis.
20   The aminopropyl CPG (AP-CPG) was derivatized to
   generate free hydroxy groups bound to this solid
   support matrix, as shown in Figure 1. To AP-CPG
   (Fluka, pore size 500A, 0.5g, 20µmol of amino
   groups) there was added α,ω-hydroxy carboxylic :
25 acid derivatives 1a, 1b or 2 (250µmol) and
   dimethylaminopyridine (DMAP) (30.5mg, 250µmol) in
   2ml of dimethyl formamide (DMF). This was either
   shaken for 3 h or left standing for 16 h. The CPG
   was then washed (DMF, CH_2Cl_2) and dried. The
30 degree of functionalization is quantitated by
   spectrophotometric assay of dimethoxytrityl
   (λ=507nm, ϵ=66500 M^{-1}) or pixyl (λ= 445 nm,
   ϵ = 4770 M^{-1}) cation released on acid treatment
   of a small amount of CPG. Residual amino groups

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(approximately 10-20%) are then acetylated by
treating the CPG with acetic anhydride (Ac$_2$O) (0.5
ml, 2.5 mmol) and DMAP (50 mg, 0.4 mmol) in pyridine
(2 mL) for 15 min. No significant residual amino

5 group was detected. The CPG was then treated with 3%
dichloroacetic acid in CH$_2$Cl$_2$ (2x5 min) and
washed with CH$_2$Cl$_2$.
    Alternatively, γ-butyrolactone was used to
derivatize the CPG. In this regard, CPG (0.5g) and

10 γ-butyrolactone (3 ml) were placed in an oven at
60°C for 7 days.
    This modified solid support was then used for
peptide synthesis. In order to couple the first
amino acid (formation of an ester linkage), a high

15 concentration of active amino acid was used. Thus,
the derivatized CPG (100mg, containing 2.7 μmol of
hydroxy functionality) was reacted with a solution of
N-BOC-alanine symmetrical anhydride and DMAP (0.2 M
in each) in DMF (2 mL) for 20 hr. Residual hydroxy

20 groups were acetylated as before using Ac$_2$O/DMAP
and the alanines deprotected to give the free amino
group (25 μmol/g). The Boc group was removed from
the first amino acid by treatment with 90%
trifluoroacetic acid (TFA)/H$_2$O (30 min), followed

25 by washing (CH$_2$Cl$_2$), neutralization (20%
triethylamine/CH$_2$Cl$_2$), washing (CH$_2$Cl$_2$) and
drying. Further peptide synthesis was then carried
out on a manual CRB peptide synthesizer, using
standard Fmoc chemistry, by utilizing a fourfold

30 molar excess of Fmoc-amino acid pentafluorophenyl
ester and 1-hydroxybenzotriazole (HOBt) in DMF.
Briefly, the CPG substrate was reacted with
N-α-FMOC-N-ε-BOC- Lys pentafluorophenyl ester in
DMF (2 mL) in the presence of HOBt for 30 min. The

reaction was quantitative by ninhydrin assay. The
FMOC group was then removed with 20% piperidine in
DMF (1 x 3 min, 1 x 7 min). Subsequent couplings
were carried out in the same way, alternating the
5 lysine residues with alanines (using FMOC-Ala
pentafluorophenyl ester) to synthesize $(AlaLys)_5Ala$.

EXAMPLE 3:
     Synthesis of an Oligonucleotide-Peptide
10 Conjugate:
     The peptide synthesized according to Example 2
was used as the starting material for oligonucleotide
synthesis.
     The terminal amino group of the peptide was
15 deprotected by 20% piperidine/DMF, and the CPG
reacted with α,ω-hydroxycarboxylic acid linkers
1a, 1b or 2 (0.2 mmol) and 1-hydroxybenzotriazole
(0.2 mM) in DMF (0.5 ml) for 16 h (see Figure 2).
Residual amino groups were acetylated and the CPG
20 used for DNA synthesis using an Applied Biosystems
380A Automated DNA Synthesizer.
     Alternatively, γ-butyrolactone was used as a
linker instead of 1a, 1b or 2. In this instance, the
CPG (100mg) was reacted with 2 ml of
25 γ-butyrolactone at 60°C for 7 days.
     The DMTr or Px protecting groups were removed
from the linker group, and oligonucleotide synthesis
commenced using methyl N,N-diisopropyl nucleoside
phosphoramidites (Beaucage and Caruthers, Supra).
30 Briefly, phosphoramidites are coupled as a 0.1M
solution in dry acetonitrile, in the presence of 0.5
M tetrazole. This is followed by acetylation of
unreacted hydroxy groups (AC_2O/DMAP), oxidation of
the phosphite triester to the phosphate (I_2/H_2O)

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and the detritylation (DCl/CH_2Cl_2) prior to
coupling of the next nucleoside phosphoramidite. The
first phosphoramidite was coupled onto the terminal
aliphatic hydroxyl group. Oligonucleotide synthesis

5   was continued and the 30mer oligodeoxyribonucleotide
d(GGGCTTCACAACATCTGTGATGTCAGCAGG) (KPIB),
complementary to part of the mRNA encoding mouse
kallikrein, was synthesized on this solid support.
The average coupling yield, by trityl assay, was over

10 99%.
        In order to deprotect the conjugate, the solid
support was removed from the automated synthesizer
and treated with $PhSH/Et_3N/CH_3CN$ 1:1:2 for 2 hr
to remove the methyl protecting groups on the

15 phosphotriesters. The BOC protecting groups on the
lysine residues (and also the 5'-DMTr group) were
removed with a 5 min treatment with 90%
trifluoroacetic acid/10% ethanedithiol, followed by
neutralization with 20% Et_3N/CH_2Cl_2. The ester

20 group attaching the C-terminal amino acid to the
solid support was then cleaved with conc. aq. NH_3
(4 hr), and the solution heated at 55°C for a further
16 hr to remove the nucleoside amino protecting
groups.

25   Figure 3 shows the pattern obtained following
$5'-^{32}P$ end labelling of the crude product with
$[\gamma-^{32}P]$-ATP using polynucleotide kinase and
electrophoresis on a 20% polyacrylamide gel
containing 7M urea. The oligonucleotide-peptide

30 hybrid runs slower than the normal KPIB and is the
major component of the product mixture. A similar
pattern is seen when the unlabelled reaction is run
and the DNA visualised on the gel by UV shadowing.
The pure hybrid product was obtained by preparative

30

gel electrophoresis on a 10% gel, or by HPLC.

Amino acid analysis of the product gave the expected ratio of 6 Ala:5 Lys, with 1 mol of (AlaLys)$_5$Ala polyamide per mole of KPIB. The

5 product was resistant to snake venom phosphodiesterase (blocked 3'-end) and was only partially digested (10 nucleotides from the 5'-end) by spleen phosphodiesterase (as assessed by HPLC analysis of the digests). It appears that the

10 presence of the positively charged polyamide at the 3'-end inhibits phosphodiesterase digestion in this region of the molecule. P$_1$ nuclease digested the conjugate to its component nucleoside and nucleotides.

DNA synthesis was performed with both methyl and

15 cyanoethyl protected nucleoside phosphoramidites according to standard procedures (Beaucage and Caruthers (1981) Tetrahedron Lett., 22, pp. 1859-1862). In these synthetic procedures a 60 to 120 second capping step with DMAP/Ac$_2$O was employed

20 after addition of each nucleoside phosphoramidite to the support matrix.

Amino acid analyses were carried out on a Beckman System 6300 Amino Acid Analyser. Spectrofluorimetric measurements were carried out on a Perkin-Elmer LS-5

25 Luminescence Spectrometer. HPLC was carried out on an Altex system, using a. Vydac C$_{18}$ 5μ 25 cm x 4.6 mm column. Buffers used were A 0.1 M triethylammonium acetate, pH 7.0 and B, 0.1 M triethylammonium acetate containing 45% CH$_3$CN, at

30 pH 7.0.

EXAMPLE 4:

Synthesis of a Polyamide Containing Synthetic Amino Acids:

The use of non-peptide amino acids introduces

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greater flexibility in the design of the molecular
architecture of the polyamide. Any
α,ω-aminocarboxylic acid may act as a spacer
between naturally occuring amino acids. The readily

5   available 6-aminohexanoic acid (HAhaOH) was chosen as
the standard unit, but any other similar amino
carboxylic acid may be used. Initially, the N-Fmoc
pentafluorophenyl active ester derivatives 3 and 4
were synthesized and used successfully in the

10 standard Fmoc peptide synthesis methodolgy. The
dimer 4 could be prepared in good yield from the
monomer 5 and 6-aminohexanoic acid. The acid 6 was
also used directly in solid phase synthesis by
utilizing the BOP (benzotriazol-1-yloxy-

15 tris-(dimethylamino)- phosphonium hexafluoroborate)
methodology (Castro, Supra). In the synthesis a
fourfold molar excess of 6, BOP reagent,
N-methylmorpholine and HOBt were used in DMF. This
proved to be a very efficient way of introducing this

20 spacer without having to make the active ester.

     To illustrate the synthesis of an oligonucleotide
containing a single primary aliphatic amino group
well removed from the oligonucleotide part, the
polyamide Aha$_4$Lys(Boc)Ala was synthesized using the

25 methodology described in Example 2 except that four
equivalents of N-Fmoc amino acid active ester 4 was
used, and utilizing two couplings of the active ester
4 to provide spacing from the oligonucleotide. A 30
mer oligonucleotide complementary to part of the

30 mouse kallikrein mRNA (KPIB) was then synthesized,
after addition of the linker 2 according to the
method of Example 3. The conjugate was deprotected
as previously described.

     In an alternative method Boc,chemistry was used

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for the synthesis of the polyamide. We used
α-Boc-ε-FmocLysOPfp (or the corresponding
carboxylic acid with BOP methodology) as the lysine
derivative and BocAha$_2$OPfp as the spacer. A four
5 fold molar excess of the active amino acid and HOBt
in DMF for 0.5 h was used. The protected
polyamide-oligonucleotide hybrid was deprotected in
exactly the same manner as a normal oligonucleotide,
to give the same product as previously described. In
10 this case the Fmoc protecting group on the lysine
residue is cleaved during the ammonia deprotection
step.

   The Fmoc methodology was also used to synthesize
a much longer polyamide, containing ten lysine
15 residues. This was Ala(LysAha$_4$)$_9$LysAla which was
synthesized in the same manner as Aha$_4$LysAla (Fmoc
methodology) using a manual peptide synthesizer.
Following the synthesis of KPIB on this substrate,
the normal deprotection protocol (see Example 3) gave
20 a product that on PAGE analysis gave a major band
running slower than KPIB. PAGE purification of this
band gave the product, which had a good amino acid
analysis.

25 EXAMPLE 5:
   Preparation of Labelled Conjugates:
   Reporter groups were introduced at four different
stages: (I) after polyamide synthesis; (II) after
addition of the linking synthon; (III) after
30 oligonucleotide synthesis; and (IV) after
deprotection and purification of the hybrid.
   (I)   Biotin and fluorescein synthons were attached
         at this stage following removal of the Boc
         protecting groups on the lysine side chains.

Biotin was coupled using a 0.2 M solution of each of biotin, BOP reagent, HOBt and N-methylmorpholine in DMF for 30 min. This coupling can also be done during polyamide synthesis after the addition of each Lys(Boc) residue. Alternativey, biotin can be added as an active ester, such as the succinimide ester. Fluorescein was coupled at this stage by reaction with a 0.3 M solution of $\underline{7}$ and HOBt in DMF for 3 d. Residual amino groups were acetylated. Since the subsequent piperidine treatment removes the isobutyryl protecting groups on the fluorescein, it was reacylated with DMAP/isobutyric anhydride in pyridine for 16 h.

(II) Coupling a label can be carried out at this stage if the lysine side chain protecting groups are Fmoc and Boc methodology was used for polyamide synthesis. Following removal of the ε-Fmoc group, biotin or fluorescein is added in the same manner to that in stage (I).

(III) The lysine side chain Boc protecting groups were removed as described previously (hybrid deprotection) and the label attached. Biotin is added by reacting with biotin N-hydroxysuccinimidyl active ester (20 equiv) and HOBt (20 equiv) in DMF (0.5 mL), for 16 h. Fluorescein is added as in stage (I) - but reacylation of the fluorescein is not necessary.

(IV) Biotin was added to the single lysine containing hybrid using standard methods. Fluorescein can also be added by using fluorescein isothiocyanate (FITC).

EXAMPLE 6:

Fluorophore conjugation to the oligonucleotide-polyamide conjugate:

The fully protected oligonucleotide-polyamide
5 conjugate:

CPG-SL-Ala]Lys(Boc)Aha]$_9$Lys(Boc)Ala-SL-KPIB

where Aha is the 6-aminohexanoic acid residue

$(-\overset{\overset{\text{O}}{\|}}{\text{C}}(CH_2)_5-NH)$, SL is a linkage derived from
10 linkers 1a or 1b, and and KPIB is the oligonucleotide
whose sequence was described above, was synthesized,
on 30 mg of CPG according to Example 4. The
conjugate was first treated with 90% trifluoroacetic
acid/10% ethanedithiol for 5 minutes, in order to
15 remove the Lys side chain Boc protecting groups,
followed by 20% triethylamine/CH_2Cl_2 to
neutralize the formed primary amino groups. The
conjugate was then reacted with of 40 fold excess of
diisobutyrylcarboxy fluorescein pentafluorophenyl
20 ester (107 mg) and 1-hydroxybenzotriazole (26mg) in
0.5 ml DMF over 3 days. The resulting labelled
compound was then deprotected. On polyacrylamide gel
electrophoresis the labelled compound gave a strongly
fluorescent band running at a similar position to the
25 xylene cyanol dye. The band was cut out and purified
to give the fluorescein labelled oligonucleotide-
polyamide conjugate.

The conjugate CPG-SL-AlaLys(Boc)Aha$_4$-SL-KPIB
was also synthesized according to the methods of
30 Example 4, and labelled with fluorescein according to
Example 5, stage 3. The labelled conjugate was then
purified by gel electrophoresis according to methods
known per se in the art.

EXAMPLE 7

Purification of Conjugates:

Purification of most of the labelled and
unlabelled hybrids was carried out by polyacrylamide
5 gel electrophoresis (PAGE) using 10% gels.
Purification of the single lysine containing hybrid
prepared by Boc chemistry was conveniently carried
out by reverse phase HPLC on a Vydac C_{18} column,
since the 5'-DMTr group of this hybrid is intact.
10 The DMTr-containing hybrid was initially purified
using the following conditions: isocratic at 33.3% B
for 20 min, and then a gradient to 66.6% B over 30
min. DMTr-KPIB-SL-Aha$_4$LysAla elutes at 44.0 min.
Detritylation of the eluate (equal volume of acetic
15 acid, 15 min) and rechromatographing on a gradient of
0 to 66.6% B over 30 min gave the pure product,
eluting at 26.0 min. The fluorescein containing
hybrids may be partially purified, mainly to remove
any free fluorescein and other low molecular weight
20 material by passage through a column of Sephadex G-25
Fine (2 g) in H_2O. The high molecular weight
fraction (0.5 mL) eluting at the void volume was
collected and dialyzed exhaustively against H_2O.

25 EXAMPLE 8

Hybridization of Conjugates to Target Sequences:

The following biotinylated conjugates were
prepared according to the methods of Example 6.

A. KPIB-LL-Aha$_4$Lys(Biotin)Ala (labelling at stage
30   IV)

B. KPIB-SL-[Aha$_2$Lys(Biotin)]Ala (stage II)

C. KPIB-SL-[AhaLys(Biotin)]$_{10}$Ala (stage III)

D. KPIB-SL [Aha$_2$Lys(Biotin)]$_{10}$Ala (stage (III)

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LL and SL stand for the linkages derived from linkers
2 and 1 respectively.
KPIB is the 30mer mouse kallikrein probe.

     Figure 4 shows the result obtained when
5   conjugates A to D (corresponding to lanes A to D)
were used to hybridize to dot blots containing a pUC
plasmid with a mouse kallikrein cDNA Insert. The dot
blots contained a 3.7 kb plasmid derived from pUC and
containing a 1 kb mouse renal kallikrein cDNA
10   insert. The negative control (NC) was a similar pUC
plasmid containing the metallothionein II_A gene
promoter spliced with the chloramphenicol acetyl
transferase (CAT) structural gene. The
nitrocellulose filters were prehybridized at 42°C for
15   6.5 h in 10 mL of hybridization buffer (0.75 M NaCl,
0.075 M sodium citrate, 25 mM NaH_2PO_4, 25 mM
Na_2HPO_4, 10 mM tetrasodium pyrophosphate, 0.1 mM
disodium adenosine triphosphate, 25 mg/L salmon sperm
DNA, 0.01% w/v Ficoll, 0.01% polyvinylpyrrolidone,
20   0.01% bovine serum albumin, 20% formamide), the probe
(100 ng) was then added and it was allowed to
hybridize at 42°C overnight. The filters were then
washed four times, for ten minutes each, at 42°C in
0.2 x SSC (0.03 M NaCl, 0.003 M sodium citrate). The
25   signal was detected using a BRL(Bethesda Research
Labs) BluGENE kit, which employs a streptavidin-
alkaline phosphatase conjugate that binds to biotin,
and a subsequent development reaction in which the
enzyme acts on substrates (nitro blue tetrazolium
30   (NBT) and 5-bromo-4-chloro-3-indoyl phosphate (BCIP)
to generate an insoluble dye precipitate.

     It is evident that there is no significant
difference in signal between hybrids that had the
biotin coupled either at stage (IV) (lane A) or stage

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(II) (lane B). This indicates that the conditions of
oligonucleotide synthesis do not adversely effect the
biotin residue so that it is not able to bind to the
streptavidin conjugate. Furthermore, the hybrids
5   containing ten biotin residues, introduced at stage
(III) (lanes C and D) give a signal that is
approximately ten tines stronger than that of the
singly labelled hybrid. There does not appear to be
a significant difference in the streptavidin binding
10 affinity of the multiply labelled hybrid with single
Aha spacers between the labels (lane C) or double Aha
spacers (lane D). The relative proximity of the
biotin residues therefore, does not appear to effect
the ability of the streptavidin-alkaline phosphatase
15 complex to bind to the biotin residues to a
significant degree. Taking the negative control (NC)
(pUC with a non-related insert) into account, the
sensitivity of the singly labelled probes is 0.5 ng
(220 amol) and the multi-labelled probes 0.05 ng (22
20 amol) by this dot blot method.
        · These biotinylated probes were also used to
hybridize into tissue sections.
        Hybridization histochemical analysis was
performed according to the methods of Coghlan, J.P.
25 et al ((1985) Anal. Biochem. 149, pp. 1-28).
Briefly, 6μm frozen sections were fixed with 4%
formaldehyde in 0.1M phosphate pH 7.2 for 5 min. and
prehybridized for 10 min. (in 50 mM sodium phosphate
pH 7.0, 5.0 mM EDTA, 0.02% ficoll, 0.02% bovine serum
30 albumin, 0.02% polyvinyl pyrrolidone and 0.01%
herring sperm DNA). Labelled conjugate was then
added to a concentration of 0.8 ng/μl.
Hybridization was for 3 days at 40°C. The sections
(attached to glass microscope slides) were then

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rinsed in 4xSSC (stock solution x 20 is 3M sodium
chloride, 0.3M sodium citrate in distilled water).
Hybridization of the conjugate was then visualised
after development under a light microscope.

5   Probe D, containing 10 biotin residues was used
to detect kallikrein mRNA in a 6 μm section of
mouse submandibular gland. As shown in Figure 5, the
probe D strongly labelled (as detected by light
microscopy) distinct regions of the submandibular

10 gland, which correspond to the granular convoluted
tubules, which are the site of expression of the
majority of mouse kallikrein genes. Hybridization
was detected using a BRL-GluGENE kit described above.

15 **EXAMPLE 9:**

        Synthesis of polyamide-oligonucleotide conjugates
having a reactive 3'hydroxyl group:
        Uridine derivatives of the formula:

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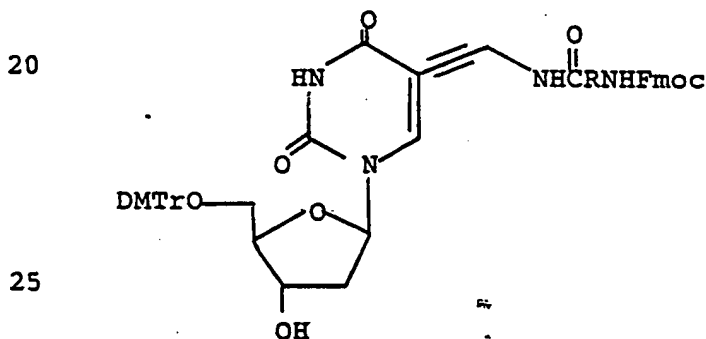
where R is alkyl or substituted alkyl,
may be synthesized according to the methods of our
co-pending Australian application No. PI 2666/87.

30   Such compounds may then be reacted with:
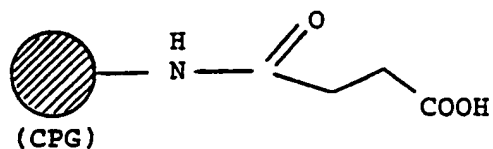
5   (there may be a spacer
    here, e.g. a polyamide)

    in the presence of DCC to give:

10

15
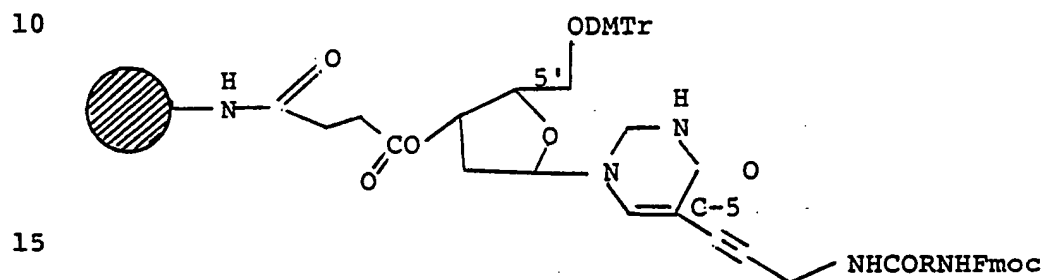
    An extended polyamide can then be synthesized on
    the C-5 arm, and following that, the oligonucleotide
20 synthesized on the 5'-hydroxyl group of the
    nucleoside.
    These uridine derivatives are useful in
    situations where extensions to the 3'OH are required,
    for example in DNA sequencing. In such situations,
25 the ester group linking the nucleoside to the CPG may
    be conveniently cleaved.

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Claims:

1.   A oligonucleotide-polyamide conjugate of the
formula X-L-Y, where X is a polyamide, Y is an
oligonucleotide, and L is a linker which forms a
covalent bond with the amino-terminus of the
polyamide X and the 3' phosphate group of the
oligonucleotide Y.

2.   A conjugate according to claim 1, wherein the
linker L is a bifunctional linker having a first
reactive group which forms a bond with the amino
terminus of a polyamide, and a second reactive group
which forms a bond with the 3' phosphate of an
oligonucleotide.

3.   A conjugate according to claim 1 or claim 2,
wherein the polyamide X is a peptide comprised of
naturally occuring amino acids.

4.   A conjugate according to any preceding claim,
wherein the polyamide is comprised of synthetic
α,ω amino carboxylic acids, or a combination of
naturally occuring amino acids and synthetic α,ω
aminocarboxylic acids.

5.   A conjugate according to any preceding claim,
wherein the polyamide contains one or more reporter
groups.

6.   A conjugate according to claim 5, wherein the
reporter groups are selected for fluorophores,
biotin, enzymes, or colloidal compounds.

7.   A conjugate according to claim 6, wherein the
reporter groups are selected from fluorescein,
teramethyl rhodamine, Texas Red, coumarins, carbonic
anhydrase, urease, horse radish peroxidase,
dehydrogenases and/or colloidal gold or silver.

8.   A conjugate according to any one of claims 1

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to 4, wherein the polyamide is antigenic and is
detectable by antibodies which bind thereto.

9. A polyamide having the formula Z-X-L, where Z
represents a solid phase matrix, X represents a
polyamide linked through its C-terminus to the said
solid phase matrix, and L represents a bifunctional
linker having a first reactive group which is
attached to the N-terminus of the polyamide, and a
second reactive groups which is capable of forming a
bond with the 3' phosphate of a nucleotide.

10. A polyamide according to claim 9, which
contains one or more reporter groups as claimed in
claims 6 or 7.

11. A method for the production of an
oligonucleotide-polyamide conjugate comprising the
step of linking the 3' terminal phosphate of a
preformed oligonucleotide or nucleotide to the amino
terminus of a preformed polyamide.

12. A method for the synthesis of an
oligonucleotide-polyamide conjugate as claimed in any
one of claims 1 to 8, the said method comprising the
steps of:

(a)   reacting the C-terminus of a first amino
acid or a unit of amino acids (linked together by
amide bonds) with a support matrix to form a bond
therebetween;

(b)   thereafter sequentially reacting the support
matrix with one or more amino acids, according to
well-known solid-phase peptide synthetic techniques
to form a polyamide;

(c)   reacting the support matrix-polyamide with a
first reactive group of a linker to form a bond
between the amino terminus of the polyamide and the
linker;

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(d)   reacting the product of step (c) with a
first nucleotide to form a bond between a second
reactive group on the linker and the 3' phosphate of
the nucleotide;

(e)   thereafter sequentially reacting the support
matrix with one or more nucleotides, according to
well-known solid phase oligonucleotide synthetic
methods to form an oligonucleotide; and

(f)   optionally cleaving the
oligonucleotide-polyamide conjugate from the support
matrix and removing any protecting groups associated
with reactive groups of the polyamide or
oligonucleotide, and purifying the resulting
conjugate.

13.   A method for determining the presence and
location in animal or plant tissue of a specific
polynucleotide population which comprises:

(a)   preparing a section of the tissue to be
examined;

(b)   hybridizing the tissue section with an
oligonucleotide-polyamide conjugate according to any
one of claims 1 to 8, wherein the oligonucleotide
portion of the conjugate is complementary to a
portion of a target polynucleotide;

(c)   removing unhybridized probe material from
the tissue section; and.

(d)   detecting or identifying the locations in
the tissue section where labelling by hybridization
of the conjugate has occurred.

14.   A method for detecting a specific
polynucleotide immobilized to or otherwise associated
with a support matrix, said method comprising
contacting the support matrix with an oligo-
nucleotide-polyamide conjugate according to any one

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of claims 1 to 8, wherein the olignucleotide portion
of the conjugate is complementary to a portion of the
target polynucleotide, and thereafter detecting
hybridization of the conjugate to the support matrix.

15. A method for detecting the presence or
absence of a specific viral, bacterial or other
polynucleotide in a biological sample, comprising
contacting the nucleic acids of the sample with an
oligonucleotide-polyamide conjugate according to any
one of claims 1 to 8, wherein the oligonucleotide
portion of the conjugate is complementary to a
portion of a target polynucleotide, and thereafter
detecting whether hybridization of the conjugate has
occurred.

16. A method for the treatment of a viral,
bacterial or other disease, characterised in that it
comprises administering to a patient in need of such
treatment a therapeutically effective amount of an
oligonucleotide-polyamide conjugate according to any
one of claims 1 to 8, and further characterised in
that the oligonucleotide portion of the conjugate is
an anti-sense oligonucleotide which is complementary
to a specific viral, bacterial or other
polynucleotide, such that transcription or
translation of the specific polynucleotide is blocked.

17. A diagnostic kit for detecting a desired
polynucleotide, which comprises an oligonucleotide-
polyamide conjugate according to any one of claims 1
to 8, wherein the oligonucleotide portion of the
conjugate is complementary to a portion of the
desired polynucleotide; and reagents for detecting
hybridization of the conjugate.

18. A diagnostic kit according to claim 17 for
use in determination of the presence and location in

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animal or plant tissue of a specific polynucleotide
population, which additionally comprises reagents for
tissue section preparation.

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FIG. 1.

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FIG. 2.

3/5

1   2
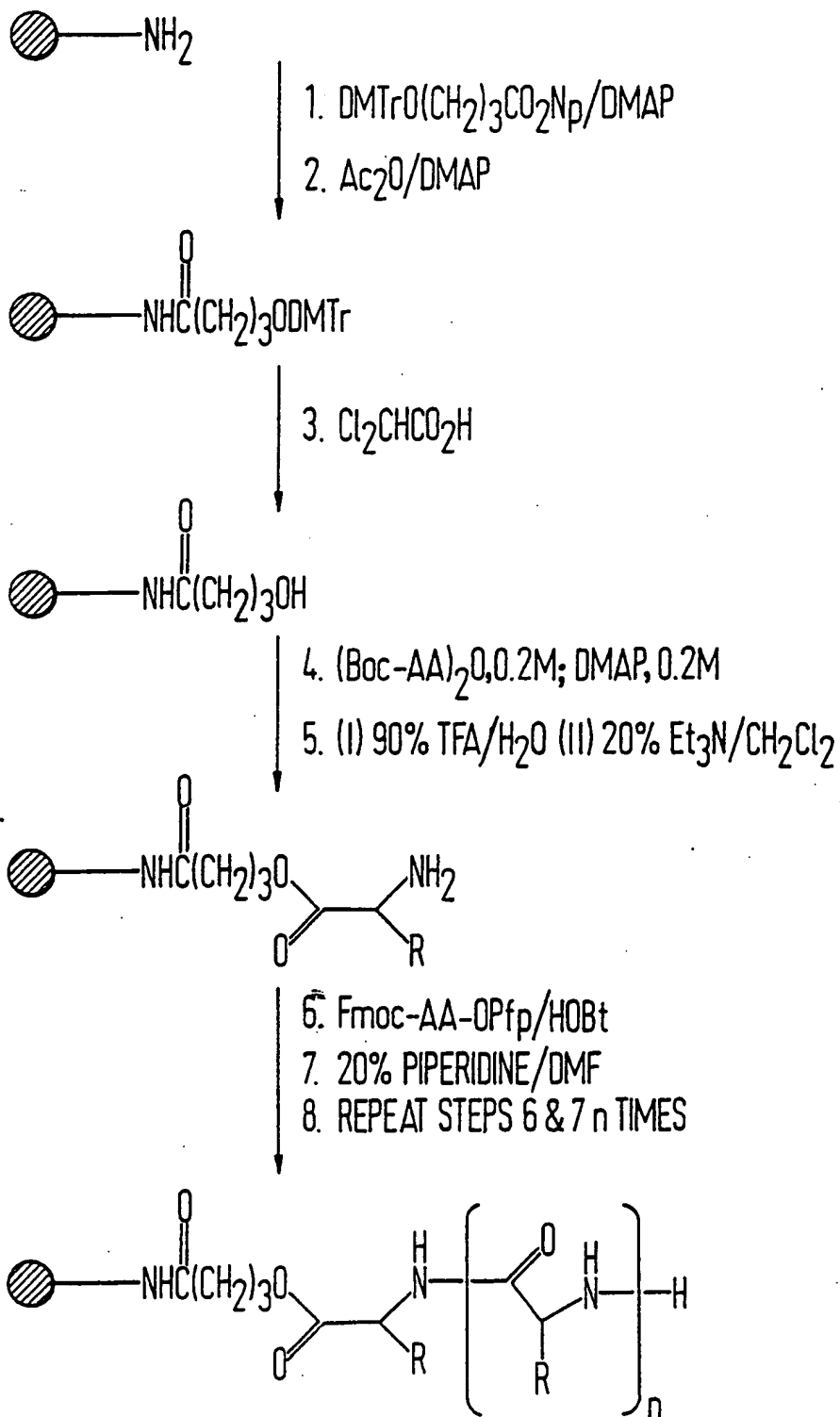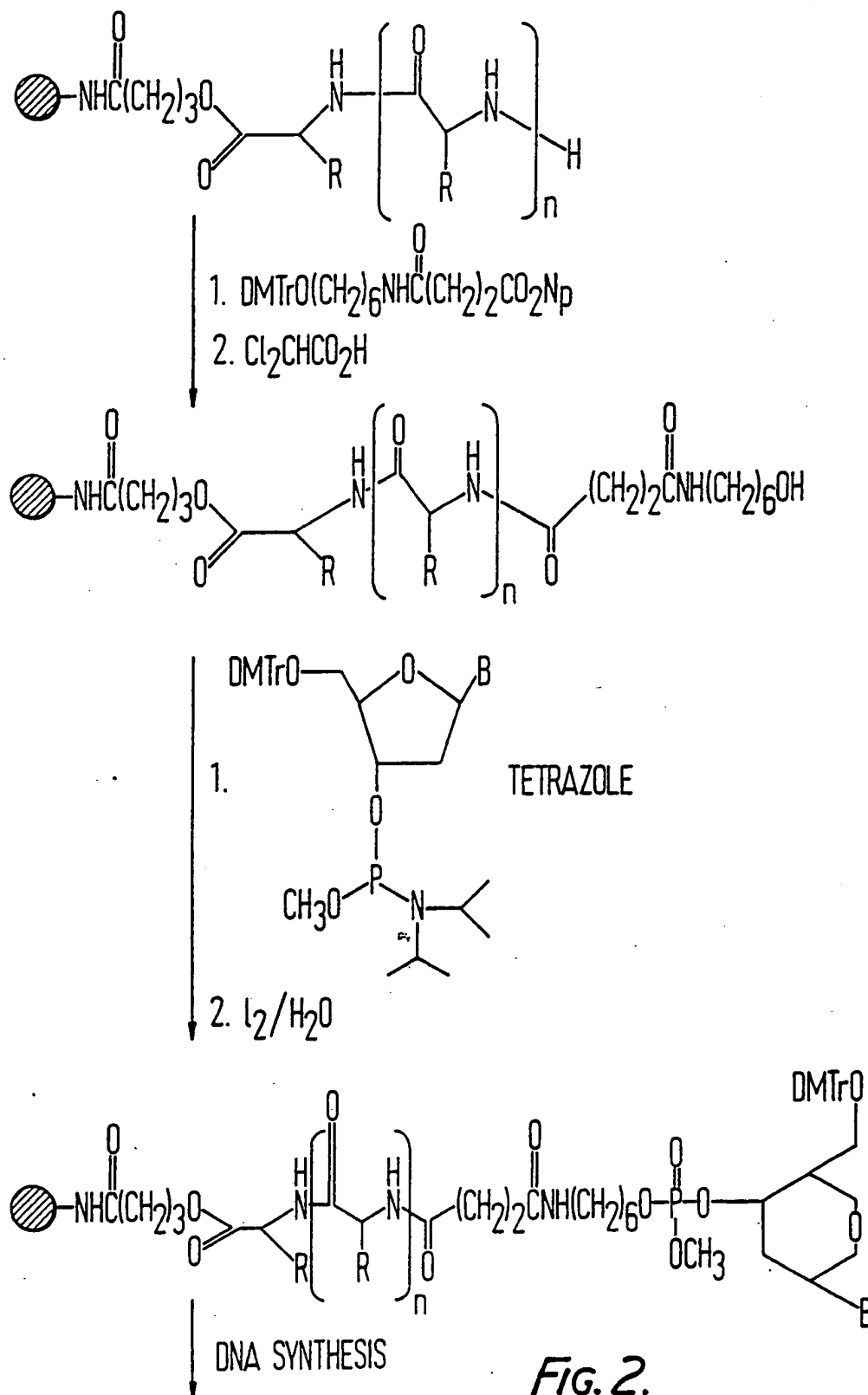
Fig. 3.
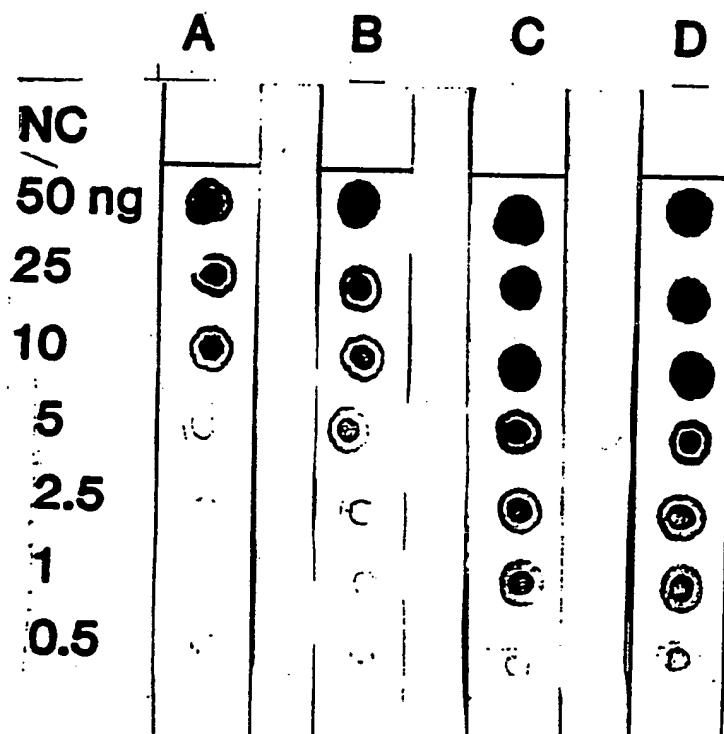
FIGURE 4

FIGURE 5

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] CO8G 69/42, 69/08, CO7H 21/04, C12Q 1/68, G01N 33/53, 33/571, 33/569, 33/532, 33/533, CO7K 7/02, 7/06, 17/08

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	WPI, WPIL, USPA : C12Q 1/68, CO7H 21/02, 21/04 (Derwent Databases)

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [8]

AU : CO7H 21/02, 21/04, G01N 33/53, CO7K 7/02, 7/06, CO7K 15/18, 17/08;
CO7G 7/00, CO7C 103/52
Chem Abstracts : Keywords: Oligonucleotide & Polypeptide, Peptide, Polyamine, Protein

III. DOCUMENTS CONSIDERED TO BE RELEVANT *

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
P,X,Y	Haralambidis, J. et al. "A new generation of non-radioactive DNA probes : oligonucleotides containing a multifunctional 3'-polyamide moiety", Nucleic Acids Research, Volume 20, issued 1988 (IRL Press Limited, Oxford, England) see pages 115 to 116.	(1-15,17,18)
P,Y	Chu, B.C.F. and L.E. Orgel "Ligation of oligonucleotides to nucleic acids or proteins via disulfide bonds", Nucleic Acid Research, Volume 16, No.9, issued 1988 (IRL Press Limited, Oxford, England) see pages 3671 to 3691.	(1-3,8,9,12-15)
Y	Chu, B.C.F. et al. "Derivatization of unprotected polynucleotides", Nucleic Acids Research, Volume 11, No.18, issued 1983 (IRL Press Limited, Oxford, England) see pages 6513 to 6518.	(1-3,8,9,12-15)
P;X	Chemical Abstracts, Volume 108, issued April 1988 (Columbus; Ohio, U.S.A.) M. Lemaitre et al. "Biological activities of oligonucleotides linked to poly (L-lysine)", see page 384, column 1, abstract no. 127810t, Nucleosides Nucleotides 1987, 6 (1-2), 311-315. (continued)	(1,3,9,11)

* Special categories of cited documents: [10]
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
9 February 1989 (09.02.89)	(20-02-89) 20 FEBRUARY 1989
International Searching Authority	Signature of Authorized Officer
Australian Patent Office	M. ROSS

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION C NTINUED FROM THE SECOND SHEET

P,X	EP,A, 251283 (WAKUNAGA SEIYAKU KABUSHIKI KAISHA; YAMANE, A.W.S. et al) 7 January 1988 (07.01.88)	(1-3,5-15,17, 18)
X,Y	EP,A, 154884 (MOLECULAR DIAGNOSTICS, INC.; DATTAGUPTA, N. et al) 18 September 1985 (18.09.85)	(1-3,5-15,17, 18)
Y	EP,B, 187332 (MOLECULAR DIAGNOSTICS, INC.; DATTAGUPTA, N. et al) 15 July 1986 (15.07.86)	(1-3,5-8)
X	AU,A, 16179/83 (ENZO BIOCHEM INC.; ENGELHARDT, D.L. et al) 5 January 1984 (05.01.84)	(1-3,5-15, 17,18)
X	AU,A, 46484/85 (BOEHRINGER MANNHEIM G.M.B.H.) 6 March 1986 (06.03.86) (continued)	(1-3,5-8,11, 13-15,17,18)

V.[X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.[X] Claim numbers ___16___, because they relate to subject matter not required to be searched by this Authority, namely:

It involves a method of treatment of a human or animal which is excluded subject matter.

2.☐ Claim numbers _____, because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.☐ Claim numbers _____, because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI.☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this International application as follows:

1.☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2)) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Chemical Abstracts, Volume 103, issued October 1985 (Columbus, Ohio, U.S.A.), D. Porschke et al., "The conformation of single stranded oligonucleotides and oligonucleotide-oligopeptide complexes from their rotation relaxation in the nanosecond time range" see page 309, column 1, abstract no. 137328c, J. Biomol. Struct. Dyn., 1985, 2(6), 1173-84.	
A	US,A, 4388306 (FIELD, A.K. et al) 14 June 1983 (14.06.83)	

This Annex lists the known "A" publication level patent family
members relating to the patent documents cited in the above-mentioned
international search report. The Australian Patent Office is in no way liable
for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members					
EP 251283	JP	63008396				
EP 154884	AU	39434/85	CA	1222706	DK	1108/85
	ES	541077	ES	8606653	FI	850925
	IL	74539	JP	60226900	NO	850769
	NZ	211366	US	4748111	ZA	8501797
EP 187332	AU	52146/86	DK	95/86	ES	550736
	ES	8800989	FI	860077	JPA2	61164160
	ZA	8600164				
AU 16179/83	CA	1223831	DK	2911/83	DK	1306/84
	DK	1307/84	EP	97373	EP	285057
	EP	285058	EP	285950	EP	286898
	ES	547320	ES	523503	ES	8606903
	ES	539316	ES	8700270	ES	8700324
	ES	547319	ES	8802257	IL	69051
	JP	59062600	NO	832292		
AU 46484/85	CA	1242657	DE	3431536	EP	173251
	JP	61060697				

END OF ANNEX